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Aviva Adler  
Aviva Adler

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UTILITY PATENT APPLICATION TRANSMITTAL LETTER (SMALL ENTITY)  
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Sir:

Transmitted herewith for filing is the patent application of **Daryl W. Hochman**.

For: **METHODS AND SYSTEMS FOR ASSESSING BIOLOGICAL MATERIALS USING OPTICAL DETECTION TECHNIQUES**

- ☒ 44 pages of specification and claims
- ☒ 11 sheets of drawings (Figs. 1A-10D)
- ☒ Combined Declaration and Power of Attorney (unsigned)
- ☒ Information Disclosure Statement and PTO Form 1449, with 29 references.

CLAIMS AS FILED

For	Number Filed	Number Extra	Rate	Basic Fee
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Please direct all telephone calls to Ann W. Speckman at telephone number (206) 269-0565.  
Address all correspondence to:

Ann W. Speckman  
LAW OFFICES OF ANN W. SPECKMAN  
2601 Elliott Avenue, Suite 4185  
Seattle, Washington 98121

Respectfully submitted,

By:

  
Ann W. Speckman  
Registration No. 31,881

LAW OFFICES OF ANN W. SPECKMAN  
2601 Elliott Avenue, Suite 4185  
Seattle, Washington 98121  
Telephone: (206) 269-0565  
Attorney Docket 48000.1002u  
June 4, 1999

**METHODS AND SYSTEMS FOR ASSESSING BIOLOGICAL MATERIALS USING  
OPTICAL DETECTION TECHNIQUES**

5 Reference to Priority Application

This application claims priority from U.S. Provisional Patent Application No. 60/088,494, filed June 8, 1998, entitled METHODS AND APPARATUS FOR ASSESSING BIOLOGICAL MATERIALS USING OPTICAL DETECTION TECHNIQUES.

10

Field of the Invention

The methods and systems of the present invention employ optical, or spectroscopic, detection techniques for assessing the health, physiological condition, and viability of biological materials such as tissues, cells, and subcellular components, and may be used in both *in vitro* and *in vivo* systems. One important application of the methods and apparatus of the present invention is high throughput screening of candidate agents and conditions to evaluate their suitability as diagnostic or therapeutic agents.

Background of the Invention

20 Drug development programs rely on *in vitro* screening assays and subsequent testing in appropriate animal models to evaluate drug candidates prior to conducting clinical trials using human subjects. Screening methods currently used are generally difficult to scale up to provide the high throughput screening necessary to test the numerous candidate compounds generated by traditional and computational means. Moreover, studies involving cell culture systems and animal model responses frequently don't accurately predict the responses and side effects observed during human clinical trials.

25 Conventional methods for assessing the effects of various agents or physiological activities on biological materials, in both *in vitro* and *in vivo* systems, generally are not highly sensitive or informative. For example, assessment of the effect of a physiological agent, such

as a drug, on a population of cells or tissue grown in culture, conventionally provides information relating to the effect of the agent on the cell or tissue population only at specified points in time. Additionally, current assessment techniques generally provide information relating to a single or a small number of parameters. Candidate agents are systematically  
5 tested for cytotoxicity, which may be determined as a function of concentration. A population of cells is treated and, at one or several time points following treatment, cell survival is measured. Cytotoxicity assays generally do not provide any information relating to the cause(s) or time course of cell death.

Similarly, agents are frequently evaluated based on their physiological effects, for  
10 example, on a particular metabolic function or metabolite. An agent is administered to a population of cells or a tissue sample, and the metabolic function or metabolite of interest is assayed to assess the effect of the agent. This type of assay provides useful information, but it does not provide information relating to the mechanism of action, the effect on other metabolites or metabolic functions, the time course of the physiological effect, general cell or  
15 tissue health, or the like.

Optical techniques have been developed and used for several applications. Light scattering has been used in the past to provide measurements of osmotic water permeability in suspensions of osmotically responsive vesicles and small cells. A.S. Verkman, "Optical Methods to Measure Membrane Transport Processes," *J. Membrane Biol.* 148:99-110, 1995.  
20 Another study reported a method for the optical measurement of osmotic water transport in cultured cells. M. Echevarria, A.S. Verkman, "Optical Measurement of Osmotic Water Transport in Cultured Cells: Role of Glucose Transporters," *J. Gen. Physiol.* 99:573-589, 1992.

Optical techniques for observing nerve activity and neuronal tissue are well-  
25 established. Hill and Keynes observed that the nerve from the walking leg of the shore crab normally has a whitish opacity caused by light scattering, and that opacity changes evoked by electrical stimulation of that nerve were measurable. Hill, D.K. and Keynes, R.D., "Opacity Changes in Stimulated Nerve," *J. Physiol.* 108:278-281, 1949. Since the publication of those results, experiments designed to learn more about the physiological mechanisms underlying

the correlation between optical and electrical properties of neuronal tissue and to develop improved techniques for detecting and recording activity-evoked optical changes have been ongoing.

Intrinsic changes in optical properties of cortical tissue have been assessed by reflection measurements of tissue in response to electrical or metabolic activity. Grinvald, A., et al., "Functional Architecture of Cortex Revealed by Optical Imaging of Intrinsic Signals," *Nature* 324:361-364, 1986; Grinvald, et al., "Optical Imaging of Neuronal Activity, Physiological Reviews, Vol. 68, No. 4, October 1988. Grinvald and his colleagues reported that some slow signals from hippocampal slices could be imaged using a CCD camera without signal averaging.

A CCD camera was used to detect intrinsic signals in a monkey model. Ts'o, D.Y., et al., "Functional Organization of Primate Visual Cortex Revealed by High Resolution Optical Imaging," *Science* 249:417-420, 1990. The technique employed by Ts'o et al. would not be practical for human clinical use, since imaging of intrinsic signals was achieved by implanting a stainless steel optical chamber in the skull of a monkey and contacting the cortical tissue with an optical oil. Furthermore, in order to achieve sufficient signal to noise ratios, Ts'o, et al., had to average images over periods of time greater than 30 minutes per image.

The mechanisms responsible for intrinsic signals are not well understood. Possible sources of intrinsic signals include dilation of small blood vessels, neuronal activity-dependent release of potassium, and swelling of neurons and/or glial cells caused, for example, by ion fluxes or osmotic activity. Light having a wavelength in the range of 500 to 700 nm may also be reflected differently between active and quiescent tissue due to increased blood flow into regions of higher neuronal activity. Yet another factor which may contribute to intrinsic signals is a change in the ratio of oxyhemoglobin and deoxyhemoglobin in blood.

U.S. Patent 5,215,095 discloses methods and apparatus for real time imaging of functional activity in cortical areas of a mammalian brain using intrinsic signals. A cortical area is illuminated, light reflected from the cortical area is detected, and digitized images of detected light are acquired and analyzed by subtractively combining at least two image frames

to provide a difference image. Allowed U.S. Patent Application No. 08/474,754 discloses similar optical methods and apparatus for optical detection of neuronal tissue and activity.

U.S. Patent 5,438,989 discloses a method for imaging margins, grade and dimensions of solid tumor tissue by illuminating the area of interest with high intensity electromagnetic radiation containing a wavelength absorbed by a contrast agent, obtaining a background video image of the area of interest, administering a contrast agent, and obtaining subsequent video images that, when compared with the background image, identify the solid tumor tissue as an area of changed absorption. U.S. Patent 5,699,798 discloses methods and apparatus for optically distinguishing between tumor and non-tumor tissue, and imaging margins and dimensions of tumors during surgical or diagnostic procedures.

U.S. Patent 5,465,718 discloses a method for imaging tumor tissue adjacent to nerve tissue to aid in selective resection of tumor tissue using stimulation of a nerve with an appropriate paradigm activate the nerve, permitting imaging of the active nerve. The '718 patent also discloses methods for imaging of cortical functional areas and dysfunctional areas, methods for visualizing intrinsic signals, and methods for enhancing the sensitivity and contrast of images. U.S. Patent 5,845,639 discloses optical imaging methods and apparatus for detecting differences in blood flow rates and flow changes, as well as cortical areas of neuronal inhibition.

U.S. Patent 5,902,732 discloses methods for screening drug candidate compounds for anti-epileptic activity using glial cells in culture by osmotically shocking glial cells, introducing a drug candidate, and assessing whether the drug candidate is capable of abating changes in glial cell swelling. This patent also discloses a method for screening drug candidate compounds for activity to prevent or treat symptoms of Alzheimer's disease, or to prevent CNS damage resulting from ischemia, by adding a sensitization agent capable of inducing apoptosis and an osmotic stressing agent to CNS cells, adding the drug candidate, and assessing whether the drug candidate is capable of abating cell swelling. A method for determining the viability and health of living cells inside polymeric tissue implants is also disclosed, involving measuring dimensions of living cells inside the polymeric matrix, osmotically shocking the cells, and then assessing changes in cell swelling. Assessment of

cell swelling activity is achieved by measuring intrinsic optical signals using an optical imaging screening apparatus.

### Summary of the Invention

5           Cells from nearly every organ and tissue, of both plant and animal origin, can be dissociated into single cells, grown and propagated using cell culture techniques. Pathological cells from diseased or dysfunctional tissue can also be isolated and maintained in tissue culture. Slices of tissue or tumors may be maintained under culture conditions for prolonged periods of time and assessed according to methods of the present invention. Short-term  
10       experiments may also be conducted on living acute tissue slices that are prepared and maintained under physiological conditions. Many multicellular systems may also be maintained as functioning systems in cell culture. Healthy, pathogenic and dysfunctional cells and tissue may also be tested and observed *in situ* in animal models.

          All cells undergo physiological processes that contribute to and determine their  
15       geometrical structure and optical properties. These physiological processes include metabolic processes, volume-regulatory responses, gene expression, endocytosis, pinocytosis, ion homeostasis, immune responses, neurological activity and inhibition, responses to mechanical trauma, chemical insult, and the like. Various events, including disease states, dysfunction, inflammation, exposure to pathogens, pollutants, radiation, chemotherapy, infectious or other  
20       agents, aging, apoptosis, necrosis, oncogenesis, and the like, affect one or more of these physiological processes, producing measurable and predictable changes in the characteristic geometrical structure or optical properties of individual cells and/or cell populations.

          The methods and systems of the present invention employ optical, or spectroscopic, detection techniques to assess the physiological state of biological materials including cells,  
25       tissues, organs, subcellular components and intact organisms. The biological materials may be of human, animal, or plant origin, or they may be derived from any such materials. Static and dynamic changes in the geometrical structure and/or intrinsic optical properties of the biological materials in response to the administration of a physiological challenge or a test

agent, are indicative and predictive of changes in the physiological state or health of the biological material.

Two different classes of dynamic phenomena are observed in viable biological materials using optical detection techniques: (1) geometrical changes in the diameter, volume, conformation, intracellular space of individual cells or extracellular space surrounding individual cells; and (2) changes in one or more intrinsic optical properties of individual cells or of cell populations, such as light scattering, reflection, absorption, refraction, diffraction, birefringence, refractive index, Kerr effect, and the like. Both classes of phenomena may be observed statically or dynamically, with or without the aid of a contrast enhancing agent.

Geometrical changes may be assessed directly by measuring (or approximating) the geometrical properties of individual cells, or indirectly by observing changes in the optical properties of cells. Changes in optical properties of individual cells or cell populations may be assessed directly using systems of the present invention.

Observation and interpretation of geometrical and/or intrinsic optical properties of individual cells or cell populations is achieved in both *in vitro* and *in vivo* systems without altering characteristics of the sample by applying physiologically invasive materials, such as fixatives. Physiologically non-invasive contrast enhancing agents, such as vital dyes, may be used in desired applications to enhance the sensitivity of optical detection techniques. In applications employing contrast enhancing agents, the optical detection techniques are used to assess extrinsic optical properties of the biological materials.

Detection and analysis of the geometrical and/or intrinsic optical properties of individual cells or sample cell populations provides information permitting the classification of the physiological state of individual cells or sample cell populations. Based on analysis of the geometrical and/or optical properties of a sample cell population, the sample may be classified as viable or non-viable, apoptotic, necrotic, proliferating, in a state of activity, inhibition, synchronization, or the like, or in any of a variety of physiological states, all of which produce distinct geometrical and/or optical profiles. The methods and systems of the present invention therefore provide for identification of the physiological state of a sample population and differentiation among various physiological states.



An important application of the methods and systems of the present invention involves screening cell populations to assess the effect(s) of exposure to various types of test agents or test conditions, including drugs, hormones and other biological agents, toxins, infectious agents, physiological stimuli, radiation, chemotherapy, and the like. The effect of various test agents and conditions may be evaluated on both normal and pathological sample populations. Safety and cytotoxicity testing is conducted by exposing a sample population to a test agent or test condition and assessing the physiological state of the sample population using optical techniques at one or more time points following administration of the test agent or test condition. Such testing may be conducted on various sample populations to determine how a test agent or condition affects a desired target sample population, as well as to predict whether a test agent or condition produces physiological side effects on sample populations that are not the target of the test agent or condition.

According to a preferred embodiment, a disease state or compromised condition is simulated in biological materials prior to administration of a test agent or test condition to assess the suitability of the test agent or condition for treating the disease state or compromised condition. Exposure of sample populations to a physiological challenge, such as a change in extracellular osmolarity or ion concentration, altered oxygen or nutrient or metabolite conditions, drugs or diagnostic or therapeutic agents, a disturbance in ion homeostasis, electrical stimulation, inflammation, infection with various agents, radiation, and the like, simulates a disease state at a cellular or tissue level. Subsequent exposure of the sample populations to a test agent or condition and detection and analysis of changes in geometrical and/or optical properties of the sample populations provides information relating to the physiological state of the sample populations produced by the test agent or condition. Screening techniques may be adapted for use with various types of cell sample populations maintained *in vitro* under appropriate cell culture conditions to provide a high throughput, automated screening system. Alternatively, screening techniques may be adapted to examine cell and tissue populations using various animal models to assess the effect of a physiological challenge and/or administration of a test agent on various cell populations in animal models *in situ*.

Changes in geometrical and/or optical properties of individual cells or cell populations may be determined by reference to empirically determined standards for specific cell types, cell densities and various physiological states, or appropriate controls may be run in tandem with the test samples to provide direct comparative data. Data is collected and, preferably, stored at multiple time points to provide data relating to the time course of the effect of a test agent or condition on sample populations. Strategies for designing screening protocols, including appropriate controls, multiple samples for screening various dosages, activities, and the like, are well known in the art and may be adapted for use with the methods and systems of the present invention.

### Description of the Figures

Preferred embodiments of the methods and systems for assessing biological materials using optical detection techniques of the present invention will be described with reference to the figures, in which:

Figures 1A-1D show a partially schematic flow diagram illustrating exemplary methods and output of the methods and apparatus of the present invention with reference to *in vitro* cell populations, wherein intrinsic optical properties of an acute rat hippocampal slice maintained in a submerged perfusion chamber are monitored at intervals during a control period and an activation period, and data is processed according to methods of the present invention.

Figures 2A-2C show the effect of the agent furosemide on stimulation-evoked afterdischarge activity in a hippocampal slice comparing the field response measurements at an extracellular electrode, with images highlighting changes in optical properties. Experiments were conducted as described in Example 1.

Figure 3A illustrates an enlarged grey-scale image of an acute rat hippocampal tissue slice, and Figs. 3B-3E illustrate enlarged, pseudo-colored images acquired as described in Example 1.

Figure 4A illustrates a view of human cortex just anterior to face-motor cortex with one recording (R) and two stimulating (S) electrodes, and four sites (labeled 1, 2, 3, and 4),

where average percent changes in corresponding optical properties were determined as described in Example 2. Figs 4B-4D illustrate plots of the percent optical changes in absorption in various spatial regions shown in Fig. 4A during electrical stimulation of the human cortex. Experiments were conducted as described in Example 2.

Figures 5A2-5C4 illustrate spatial images of stimulation-induced epileptiform activity. The images show comparisons between different degrees of activation illustrating both the spatial extent and amplitude of optical changes indicative of the extent of cortical activity. Experiments were conducted as described in Example 2.

Figures 6A-6H illustrate percentage difference images in which the magnitude of optical change indicates the regions of greater cortical activity. Experiments were conducted as described in Example 2.

Figures 7A-7H illustrate percentage difference images representing a real time sequence of dynamic changes of electrical stimulation-evoked optical changes in human cortex. Experiments were conducted as described in Example 2.

Figures 8A1-8B3 illustrate functional mapping of human language (Broca's area) and tongue and palate sensory area in an awake human patient as described in Example 3. Figures 8A1 and 8B2 illustrate control percentage difference images and Figures 8A3 and 8B3 illustrate peak optical change images following cortical stimulation.

Figures 9A and 9B show time course and magnitude plots of dynamic optical changes in human cortex evoked in tongue and palate sensory areas and in Broca's area (language). Experiments were conducted as described in Example 3.

Figures 10A-10D illustrate the cranial surface of a rat, imaged through the intact cranium, and using a contrast enhancing agent to highlight areas of optical change. Experiments were conducted as described in Example 4.

#### Description of Preferred Embodiments

The following description of preferred embodiments includes detailed descriptions of specific applications, as well as specific methods and apparatus. These specific embodiments are described for purposes of illustrating the scope of the invention; the invention is not

limited to these applications. Techniques for acquiring data relating to optical properties of various types of tissues that would be suitable for use with the methods and systems of the present invention are described in numerous U.S. Patents. U.S. Patents 5,215,095, 5,438,989, 5,699,798, 5,465,718, 5,845,639 and 5,902,732 are hereby incorporated herein by reference in  
5 their entireties.

One important application of methods and systems of the present invention is to classify the physiological condition or state of biological materials based on their geometrical and/or optical properties, and to distinguish among various physiological conditions. Viable cell populations are distinguishable from non-viable cell populations in both *in vitro* cell  
10 sample populations, and *in situ* in animal models based on a comparison of geometrical and/or optical properties. Similarly, sample populations that are proliferating, or that are responding to various stimuli by mounting certain responses, such as immune responses, inflammatory responses, and the like, are distinguishable from non-responsive sample populations.

Cells or cell populations undergoing apoptosis, an active programmed cell death  
15 phenomenon, are likewise distinguishable from cells or cell populations undergoing necrosis. Necrosis may result from mechanical injury, exposure to toxins, anoxia due to impairment of the blood supply, or the like. The physiological changes observed during necrosis include swelling, clumping of chromatin, and deterioration of the organelles, followed by lysis with release of the cell contents, which are then phagocytized by macrophages. The cytological  
20 changes associated with apoptosis are very different and include an early condensation of chromatin and degradation of DNA, and cell volume decreases, with the cell membrane and the organelles remaining intact. Apoptotic cells ultimately fragment into several membrane-bounded globules that are phagocytized by neighboring cells. Neutrophils and macrophages are not involved in the terminal stages of apoptotic processes to the same extent that they are  
25 in necrotic processes.

Identifying cell populations undergoing apoptosis is important for numerous reasons. Certain genes responsible for regulating apoptosis play a role in cancer, and cancer therapy by irradiation, chemotherapy, and hormone treatment all induce apoptosis in tumor cells. Some cancers may, in fact, result from the down-regulation of genes that normally cause apoptosis.

Hence, methods for screening cell populations to ascertain whether or not they are apoptotic is central to gaining an insight into various pathological conditions, such as cancer. Since different agents used in cancer treatment induce apoptosis, it is likely that apoptotic pathways are indicative of the outcome of chemotherapy.

5           There are many other instances of apoptosis during both normal and pathological cellular activities. For example, hormones regulate apoptosis in gonadal tissues so that numbers and development of sperm and egg cells are regulated. In the immune system, apoptosis plays a clear role in the selection of lymphocytes. Immunodeficiency may be caused by lymphocyte developmental blocks which lead to apoptosis by default. Cell death in these cases is the normal, programmed response in the absence of an essential survival signal.

10           However, active induction of programmed cell death can also elicit immunodeficiency, as in acquired immunodeficiency syndrome, AIDS. Considerable evidence supports the proposition that HIV activates T cell apoptosis. In the nervous system, apoptosis plays not only a pivotal role during embryogenesis, but also occurs in the adult, generally under various pathological conditions that are accompanied by devastating consequences for the patients. Examples include Alzheimer's disease, amyotrophic lateral sclerosis (ALS) and other types of neuronal injury inflicted by ischemia, hypoglycemia or excitotoxic agents. Trauma, stroke, excitotoxicity and hypoxia are conditions of ischemia that are associated with extensive neuronal cell death in sensitive brain regions. Recent studies demonstrated that, after experimentally induced ischemia, characteristic apoptotic DNA fragmentation occurs in affected brain regions.

15           Methods and systems of the present invention for classifying cell populations as apoptotic, necrotic, viable, non-viable, and the like, are useful for identifying the physiological state of cells in both *in vitro* and *in vivo* systems, as well as for screening various test agents to ascertain whether they are useful as diagnostic or therapeutic agents.

20

25           Methods and systems of the present invention may also be used to identify physiological conditions associated with and to evaluate test agents and conditions for diagnosis and treatment of various disorders, and pathological conditions, including migraine headaches, spreading depression, epilepsy, Alzheimer's disease, multiple sclerosis, psychiatric disorders such as depression, anxiety, bipolar disorder, schizophrenia, Parkinson's disease and

other neurodegenerative disorders, inflammation, trauma, malignancies such as cancer, angiogenesis, wound healing, immune deficiencies, and the like. Test agents and conditions may also be tested for safety and efficacy for applications such as toxicology, learning and memory, bone growth and maintenance, muscle and blood systems, sensory-input systems, and the like.

Optical contrast enhancing agents useful for enhancing the sensitivity of optical detection for various types of cells, physiological states, and the like, may also be screened and identified using methods and systems of the present invention. Sample populations comprising malignant, pathological, or dysfunctional cells may be exposed to test agents, for example, to identify agents that preferentially identify and distinguish malignant, pathological, and dysfunctional cells or tissue.

According to one embodiment, methods and systems of the present invention acquire and compare data representative of one or more dimensional properties of individual cells or cell samples. Acquisition, processing and analysis of data relating to optical properties is described throughout this description. Acquisition of data relating to dimensional properties of individual cells is described below. Acquisition and analysis of data relating to dimensional properties may be achieved using the same or similar methods and apparatus described herein with reference to optical properties.

In sparsely populated cell samples, cell areas may be approximated using a single plane of focus. If it is desired to calculate volume, the z-axis (focus) can be automatically adjusted as well. For example, as an automated and controlled stage moves, the optically transparent container containing the sample population is positioned so that a series of data sets for multiple, spatially resolved areas of interest can be acquired, each image being acquired at a predetermined focal plane. The volume for each z-plane can be approximated (see algorithm below) and then the volumes for each z-coordinate added together.

General techniques for approximating cell areas and volumes, based on Doughty, S., "Calculating property for solids of revolution," *Machine Design*, pp 184-186, 10 Dec. 1981, are described below. These techniques are based on Green's theorem:

$$\oint (Pdx + Qdy) \quad \iint \left( \frac{\partial Q}{\partial x} - \frac{\partial P}{\partial y} \right) dx dy$$

(boundary)

(area)

5

Individual cells are examined using an appropriate magnifying device. Edge detection of cell boundaries is achieved using, for example, a Sobel operator. The boundary is approximated by fitting it to a plurality of straight line segments of “n” line segments by “n” nodes. The integration of the boundary may be taken as “n” line integrals as follows:

10

$$\oint_{\Gamma} (\dots) ds = \int_{X_1, Y_1}^{X_2, Y_2} (\dots) ds + \int_{X_2, Y_2}^{X_3, Y_3} (\dots) ds + \dots + \int_{X_n, Y_n}^{X_1, Y_1} (\dots) ds$$

15

There are three cases:

- Case 1: a vertical line,  $x = \text{constant}$ ;
- Case 2: a horizontal line,  $y = \text{constant}$ ;
- Case 3: an inclined line,  $y = 5_i(x - x_i) + y_i$

20

The area is thus:

$$A = \iint_A 1 dx dy$$

25

To apply Green’s theorem, the integral can be considered in the form of  $\delta Q/\delta x - \delta P/\delta y$  and appropriate functions can be devised, *e.g.*,  $P(x, y)$  and  $Q(x, y)$ . For an area calculation, consider  $Q(x, y) = 0$  so that  $\oint Q dy = 0$ , and let  $P = -y$  so that  $\delta P/\delta y = -1$ . Then, the area can be calculated as follows:

$$\begin{aligned}
A &= \iint_1 dx dy = - \iint \delta P / \delta y \, dx dy \\
&= \iint (2Q / \delta x - \delta P / \delta y) \, dx dy \\
&= \oint (P dx + Q dy)
\end{aligned}$$

(Green's Theorem)

5

$$\begin{aligned}
&= \oint P dx \\
&= - \oint y dx
\end{aligned}$$

$$\text{Case 2: } \Delta A = - y_i (x_{i+1} - x_i)$$

$$\text{Case 1} = 0$$

10

$$\text{Case 3: } \Delta A = - [ \frac{1}{2} y_i (x_{i+1}^2 - x_i^2) + (y_i - y_{i+1}) (x_{i+1} - x_i) ]$$

$$A = \sum \Delta A$$

15

For volume calculations, conventional edge detection using, for example, a Sobel operator, can be used to focus through an individual cell, which can be divided into a plurality (n) of individual, planar sections. The volume for each of the "n" sections can be calculated as  $\Delta V_i = \Delta z_i \bullet \Delta A_i$ ; and the volume of the entire cell can be approximated as:  $V = \sum \Delta V_i$ . The determination and comparison of cell areas and volumes is preferably accomplished using computer hardware and/or software implementations.

20

According to another embodiment, methods and systems of the present invention acquire and compare data representative of one or more optical properties of individual cells or areas of interest in cell sample populations. Changes in optical properties that are indicative of physiological activity and that may be detected include, for example, reflection, refraction, diffraction, absorption, scattering, birefringence, refractive index, Kerr effect, and



the like. Changes in optical properties are detected directly using photon sensitive elements and, optionally, optical elements that enhance the detected optical properties.

High resolution detection of dynamic geometrical and optical properties indicative of physiological activity may be accomplished without using dyes or other types of contrast enhancing agents according to the methods and apparatus of the present invention, as evidenced by the examples described herein. Many of the assessment techniques and apparatus of the present invention are physiologically noninvasive, in that detection and analysis of geometrical and/or intrinsic optical information does not require direct contact of the area of interest with any agents such as dyes, oils, devices, or the like. For particular applications, it may, however, be useful to administer contrast enhancing agents that amplify differences in an optical property being detected as a function of physiological activity prior to acquiring subsequent data and generating a comparison. The use of contrast enhancing agents is described in detail, with reference to optical imaging of tumor and non-tumor tissue, in U.S. Patent 5,465,718 and U.S. Patent 5,438,989, which are incorporated by reference herein in their entireties. Suitable contrast enhancing agents include fluorescent and phosphorescent materials, dyes that bind to cell membranes, optical probes that preferentially accumulate in blood or in the intracellular space, phase resonance dye pairs, and the like. Detectors appropriate for use with such contrast enhancing agents are well known in the art.

Numerous devices for acquiring, processing and displaying data representative of one or more geometrical and/or optical properties of a cell sample population in culture or an area of interest *in situ* in an animal model may be employed. One preferred device is a camera that acquires images of one or more areas of interest at predetermined time intervals that can be compared to identify areas of changes in geometrical and/or optical properties that indicate physiological activity or dysfunction. The data acquisition device preferably incorporates or is used in conjunction with a device that magnifies the area of interest, such as a microscope.

Magnification sufficient to provide resolution of individual cells is preferred. An inverted microscope such as a Nikon Diaphot 300 is suitable. For high throughput screening techniques using cell sample populations maintained under culture conditions, samples in optically transparent containers such as flasks, plates and multi-well plates, may be placed on

an automated stage that is controlled and moved in a programmed fashion to permit periodic examination of individual cells or cell populations according to a programmed schedule. For example, a multi-well culture plate having a plurality of cell samples may be placed on an automated and controllable microscope stage. The stage is controlled by an automated microcontroller so that it automatically moves into position over each culture well. A data set relating to geometrical and/or optical properties of individual cells or a cell population is acquired for each position. In this manner, the system can rapidly and systematically acquire data corresponding to many samples. The physiological environment in selected wells may be altered by exposure to a physiological challenge, test agent or test condition, and the system may continue to automatically acquire data from the same wells in each culture plate at predetermined time intervals following treatment, with data acquired from various treatment wells being compared to data acquired from various control wells or empirically determined controls.

Acquisition of data representative of one or more geometrical and/or optical properties preferably provides high spatial resolution as well, so that geometrical or optical data corresponding to a particular spatial location is acquired at various time intervals for comparison. In this fashion, data acquired from single cells or highly localized areas of interest in cell sample populations is compared to provide reliable and highly-sensitive information concerning the physiological state or condition of the sample population. High spatial resolution is provided, for example, by implementing high resolution cameras and charge coupled devices (CCDs). Apparatus suitable for obtaining such images have been described in the patents incorporated herein by reference and are more fully described below. The optical detector preferably provides images having a high degree of spatial resolution at a magnification sufficient to detect single cells. Several images may be acquired at predetermined time intervals and combined, such as by averaging, to provide images for comparison.

Various data processing techniques may be advantageously used to assess the data collected in accordance with the present invention. Comparison data may be assessed or

presented in a variety of formats. Processing may include averaging or otherwise combining a plurality of data sets to produce control, subsequent and various comparison data sets. Data may be converted from an analog to a digital form for processing, and back to an analog form for display as an image. Alternatively, data may be acquired, processed, analyzed, and output in a digital form.

Data processing may also include amplification of certain signals or portions of a data set (*e.g.*, areas of an image) to enhance the contrast seen in data set comparisons, and to thereby identify cells or cell populations undergoing changes in geometrical and/or optical properties with a high degree of spatial resolution. For example, according to one embodiment, images are processed using a transformation in which image pixel brightness values are remapped to cover a broader dynamic range of values. A "low" value may be selected and mapped to zero, with all pixel brightness values at or below the low value set to zero, and a "high" value may be selected and mapped to a selected value, with all pixel brightness values at or above the high value mapped to the high value. Pixels having an intermediate brightness value, representing the dynamic changes in brightness indicative of neuronal activity, may be mapped to linearly or logarithmically increasing brightness values. This type of processing manipulation is frequently referred to as a "histogram stretch" and can be used according to the present invention to enhance the contrast of data sets, such as images, representing changes in neuronal activity.

Data processing techniques may also be used to manipulate data sets to provide more accurate combined and comparison data. For example, for *in vivo* applications, movement, respiration, heartbeat, seizure or reflex activity may shift an area of interest during data acquisition. It is important that corresponding data points in data sets are spatially resolved and precisely aligned to provide accurate combined and comparison data. Optical markers may be fixed at an area of interest and detected as the data is collected to aid in manual alignment or mathematical manipulation of data sets. Various processing techniques are described below and in the patents incorporated herein by reference.

Comparison data may be displayed in a variety of ways. Comparison data may be displayed, for example, in a graphical format that highlights geometrical or optical differences indicative of physiological changes. A preferred technique for presenting and displaying comparison data is in the form of visual images or photographic frames corresponding to spatially resolved areas of interest. This format provides a visualizable spatial location (two- or three-dimensional) of a cell population being analyzed. To enhance and provide better visualization of high contrast areas indicating changes in geometrical and/or optical properties indicative of physiological activity or dysfunction, comparison data may be processed to provide an enhanced contrast grey scale or even a color image. A look up table ("LUT") may be provided, for example, that converts the grey scale values for each pixel to a different (higher contrast) grey scale value, or to a color value. Color values may map to a range of grey scale values, or color may be used to distinguish between positive-going and negative-going geometrical or optical changes. In general, color-converted images provide higher contrast images that highlight changes in optical properties representing physiological activity, function or dysfunction.

Systems of the present invention generally comprise an illumination source for illuminating the biological material, an optical detector for acquiring data relating to a geometrical or optical property of the biological material, and data storage and analysis and output device(s) for storing data relating to a geometrical or optical property of the biological material, comparing various data sets, and/or control data profiles, to generate comparison data relating to changes in geometrical and/or optical properties indicative of changes in the physiological state of sample populations and to provide or display the output data in a useful format.

An emr source is used for illuminating an area of interest during acquisition of data representing one or more dimensional or intrinsic optical properties of cells or tissue at an area of interest. The emr source may be utilized to illuminate an area of interest directly, as when *in vitro* cell cultures maintained in optically transparent containers are illuminated or when tissue is exposed, such as in connection with surgery, or it may be utilized to illuminate an

area of interest indirectly through adjacent or overlying tissue such as bone, dura, skin, muscle and the like. The emr source employed in the present invention may be a high or low intensity source, and may provide continuous or non-continuous illumination. Suitable illumination sources include high and intensity sources, broad spectrum and non-chromatic sources, tungsten-halogen lamps, lasers, light emitting diodes, and the like. Cutoff filters for selectively passing all wavelengths above or below a selected wavelength may be employed. A preferred cutoff filter excludes all wavelengths below about 695 nm.

Preferred emr wavelengths for acquiring data relating to intrinsic optical signals include, for example, wavelengths of from about 450 nm to about 2500 nm, and most preferably, wavelengths of the near infrared spectrum of from about 700 nm to about 2500 nm. Generally, longer wavelengths (*e.g.*, approximately 800 nm) are employed to detect cellular or tissue condition of locations beneath the surface of cells or tissue, or beneath other materials such as skin, bone, dura, and the like. cortical activity. Selected wavelengths of emr may also be used, for example, when various types of contrast enhancing agents are administered. The emr source may be directed to the area of interest by any appropriate means. For some applications, the use of optical fibers is preferred. One preferred arrangement provides an emr source through strands of fiber optic using a beam splitter controlled by a D.C. regulated power supply (Lambda, Inc.).

The optical detection methods of the present invention may also usefully employ non-continuous illumination and detection techniques. For example, short pulse (time domain), pulsed time, and amplitude modulated (frequency domain) illumination sources may be used in conjunction with suitable detectors (*see*, Yodh, A. and Chance, B., *Physics Today*, March, 1995). Frequency domain illumination sources typically comprise an array of multiple source elements, such as laser diodes, with each element modulated at 180° out of phase with respect to adjacent elements (*see*, Chance, B. et al., *Proc. Natl. Acad. Sci. USA*, 90:3423-3427, 1993). Two-dimensional arrays, comprising four or more elements in two orthogonal planes, can be employed to obtain two-dimensional localization information. Such techniques are described

in U.S. Patents 4,972,331 and 5,187,672 which are incorporated by reference herein in their entireties.

Time-of-flight and absorbance techniques (Benaron, D.A. and Stevenson, D.K., *Science* 259:1463-1466, 1993) may also be usefully employed in the present invention. In yet another embodiment of the present invention, a scanning laser beam may be used in conjunction with a suitable detector, such as a photomultiplier tube, to obtain high resolution data images, preferably in the form of an area of interest.

Illumination with a part of the infrared spectrum allows for detection of intrinsic optical signals through tissue overlying or adjacent the area of interest, such as dura and skull.

One exemplary infrared emr source suitable for detection of intrinsic optical signals through tissue overlying or adjacent the area of interest is a Tunable IR Diode Laser from Laser Photonics, Orlando, FL. When using this range of far infrared wavelengths, the optical detector is preferably provided as an infrared (IR) detector. IR detectors may be constructed from materials such as indium arsenide, germanium and mercury cadmium telluride, and are generally cryogenically cooled to enhance their sensitivity to small changes in infrared radiation. One example of an IR detection system which may be usefully employed in the present invention is an IRC-64 infrared camera (Cincinnati Electronics, Mason, OH).

The area of interest is preferably evenly illuminated to effectively adjust the signal over a full dynamic range, as described below. Nonuniformity of illumination is generally caused by fluctuations of the illumination source and intensity variations resulting from the three-dimensional nature of the tissue surface. More uniform illumination can be provided over the area of interest, for example, by using diffuse lighting, mounting a wavelength cutoff filter in front of the optimal detector and/or emr source, or combinations thereof. Fluctuation of the illumination source itself is preferably prevented by using a light feedback mechanism to regulate the power supply of the illumination source. In addition, a sterile, optically transparent plate may contact and cover an area of interest to provide a flatter, more even contour surface for detection. Fluctuations in illumination can be compensated for using

detection processing algorithms, including placing a constant shade grey image marker point at the area of interest as a control point.

The system also comprises an optical detector for acquiring a signal representative of one or more optical properties of the area of interest. Any photon detector may be employed as an optical detector. Suitable optical detectors include, for example, photo diodes, photo multiplier tubes, photo sensitive silicon detector chips, such as those provided in CCD devices, and the like. Multiple emr sources and/or multiple photon detectors may be provided and may be arranged in any suitable arrangement. Specialized detectors for detecting selected optical properties may be employed. One preferred optical detector for acquiring data in the format of an analog video signal is a CCD video camera which produces an output video signal at 30 Hz having, for example, 512 horizontal lines per frame using standard RS 170 convention. One suitable device is a CCD-72 Solid State Camera (Dage-MTI Inc., Michigan City, IN). Another suitable device is a COHU 6510 CCD Monochrome Camera with a COHU 6500 electronic control box (COHU Electronics, San Diego, CA). In some cameras, the analog signal is digitized 8-bits deep on an ADI board (analog-to-digital board). The CCD may be cooled, if necessary, to reduce thermal noise.

Data processing is an important feature of the optical detection and analysis techniques and systems of the present invention. In use, for example, a CCD apparatus is preferably adjusted (at the level of the analog signal and before digitizing) to amplify the signal and spread the signal across the full possible dynamic range, thereby maximizing the sensitivity of the apparatus. Specific methods for detecting optical signals with sensitivity across a full dynamic range are described in detail in the patents incorporated herein by reference. Means for performing a histogram stretch of the difference frames (*e.g.*, Histogram/Feature Extractor HF 151-1-V module, Imaging Technology, Woburn, MA) may be provided, for example, to enhance each difference image across its dynamic range. Exemplary linear histogram stretches are described in Green, *Digital Image Processing: A Systems Approach*, Van Nostrand Reinhold: New York, 1983. A histogram stretch takes the brightest pixel, or one with the highest value in the comparison image, and assigns it the maximum value. The

lowest pixel value is assigned the minimum value, and every other value in between is assigned a linear value (for a linear histogram stretch) or a logarithmic value (for a log histogram stretch) between the maximum and minimum values. This allows the comparison image to take advantage of the full dynamic range and provide a high contrast image that clearly identifies areas of neuronal activity or inactivity.

Noise (such as 60 Hz noise from A.C. power lines) is filtered out in the control box by an analog filter. Additional adjustments may further enhance, amplify and condition the analog signal from a CCD detector. One means for adjusting the input analog signal is to digitize this signal at video speed (30 Hz), and view the area of interest as a digitized image that is subsequently converted back to analog format.

It is important that data, such as consecutive data sets corresponding to a particular area of interest, be aligned so that data corresponding to the same spatially resolved location is compared. If data sets are misaligned prior to comparison, artifacts are introduced and the resulting comparison data set may amplify noise and edge information. Data misalignment may be caused by sample movement or motion, heartbeat, respiration, and the like. Large movements of cells in an area of interest being analyzed may require a new orientation of the detector. It is possible to compensate for small movements of cells in the area of interest by either mechanical or computational means, or a combination of both.

Real-time motion compensation and geometric transformations may also be used to align corresponding data. Simple mechanical translation of data or more complex (and generally more accurate) geometric transformation techniques can be implemented, depending upon the input data collection rate and amount and type of data processing. For many types of image data, it is possible to compensate by a geometrical compensation which transforms the images by translation in the x-y plane. In order for an algorithm such as this to be feasible, it must be computationally efficient (preferably implementable in integer arithmetic), memory efficient, and robust with respect to changes in ambient light.

For example, functional control points or numbers can be located in an area of interest and triangulation-type algorithms used to compensate for movements of these control points.



Goshtasby ("Piecewise Linear Mapping Functions for Image Registration," *Pattern Recognition* 19:459-66, 1986) describes a method whereby an image is divided into triangular regions using control points. A separate geometrical transformation is applied to each triangular region to spatially register each control point to a corresponding triangular region in a control image.

"Image warping" techniques may be employed whereby each subsequent image is registered geometrically to the averaged control image to compensate for movement. Image warping techniques (described in, for example, Wolberg, *Digital Image Warping*, IEEE Computer Society Press: Los Alamitos, CA, 1990), may be used. Image warping techniques can further indicate when movement has become too great for effective compensation and a new averaged control image must be acquired.

The data storage processing and analysis function is generally performed and controlled by a host computer. The host computer may comprise any general computer (such as an IBM PC type with an Intel 386, 486, Pentium or similar microprocessor or Sun SPARC) that is interfaced with the emr source and/or optical detector and controls data acquisition and flow, comparison computations, analysis, output, and the like. The host computer thus controls acquisition and analysis of data and provides a user interface.

The host computer may comprise a single-board embedded computer with a VME64 interface, or a standard (IEEE 1014-1987) VME interface, depending upon bus band width considerations. Host computer boards which may be employed in the present invention include, for example, Force SPARC/CPU-2E and HP9000 Model 7471. The user interface can be, for example, a Unix/X-Window environment. The image processing board can be, for example, based upon Texas Instruments' MVP and other chips to provide real-time image averaging, registration and other processing necessary to produce high quality difference images for intraoperative viewing. This board will also drive a 120 x 1024 RGB display to show a sequence of difference images over time with pseudo-color mapping to highlight tumor tissue. Preferably, a second monitor is used for the host computer to increase the overall screen real estate and smooth the user interface. The processing board (fully

programmable) can support a VME64 master interface to control data transactions with the other boards. Lastly, a peripheral control board can provide electrical interfaces to control mechanical interfaces from the host computer. Such mechanical interfaces can include, for example, the light source and optical detector control box.

A real-time data acquisition and display system, for example, may comprise four boards for acquisition, image processing, peripheral control and host computer. A minimal configuration with reduced processing capabilities may comprise just the acquisition and host computer boards. The acquisition board comprises circuitry to perform real-time averaging of incoming video frames and allow readout of averaged frames at a maximum rate bus. A VME bus is preferred because of its high peak bandwidth and compatibility with a multitude of existing VME products. The acquisition board should also support many different types of optical detectors via a variable scan interface. A daughter board may support the interfacing needs of many different types of optical detectors and supply variable scan signals to the acquisition motherboard. Preferably, the unit comprises a daughter board interfacing to an RS-170A video signal to support a wide base of cameras. Other camera types, such as slow scan cameras with a higher spatial/contrast resolution and/or better signal to noise ratio, can be developed and incorporated in the inventive device, as well as improved daughter boards to accommodate such improved cameras.

Data relating to dimensional and/or intrinsic optical properties of a sample population acquired, for example, as analog video signals, may be continuously processed using, for example, an image analyzer (*e.g.*, Series 151 Image Processor, Imaging Technologies, Inc., Woburn, MA). An image analyzer receives and digitizes an analog video signal with an analog to digital interface and performs at a frame speed of about 1/30th of a second (*e.g.*, 30 Hz or "video speed"). Processing the signal involves first digitizing the signal into a series of pixels or small squares assigned a value (in a binary system) dependent upon the number of photons (*i.e.*, quantity of emr) being reflected off tissue from the part of the area of interest assigned to that pixel. For example, in a standard 512 x 512 image from a CCD camera, there

would be 262,144 pixels per image. In an 8 bit system, each pixel is represented by 8 bits corresponding to one of 256 levels of grey.

The signal processor may include a programmable look-up table (*e.g.*, CM150-LUT16, Imaging Technology, Woburn, MA) initialized with values for converting grey coded pixel values, representative of a black and white image, to color coded values based upon the intensity of each grey coded value. Using image stretching techniques, the highest and lowest pixel intensity values representing each of the pixels in a digital image frame are determined over a region of the image frame which is to be stretched. Stretching a selected region over a larger range of values permits, for example, easier identification and removal of relatively high, spurious values resulting from noise.

The signal processor means may further include a plurality of frame buffers having frame storage areas for storing frames of digitized image data received from the A/D interface. The frame storage area comprises at least one megabyte of memory space, and preferably at least 8 megabytes of storage space. An additional 16-bit frame storage area may be provided as an accumulator for storing processed image frames having pixel intensities represented by more than 8 bits. The processor means preferably includes at least three frame buffers, one for storing the averaged control image, another for storing the subsequent image, and a third for storing a comparison image.

The signal processor may further comprise an arithmetic logic unit (*e.g.*, ALU-150 Pipeline Processor) for performing arithmetical and logical functions on data located in one or more frame buffers. An ALU may, for example, provide image (data) averaging in real time. A newly acquired digitized image may be sent directly to the ALU and combined with control images stored in a frame buffer. A 16 bit result can be processed through an ALU, which will divide this result by a constant (*i.e.*, the total number of images). The output from the ALU may be stored in a frame buffer, further processed, or used as an input and combined with another image.

Normally, areas of increased physiological activity exhibit an increase of the emr absorption capacity of the cell sample or tissue (*i.e.*, the cell sample gets darker if visible light

is used for emr illumination, or an intrinsic signal increases in a positive direction). Similarly, a reduction in physiological activity generally corresponds to a decrease of emr absorption capacity of the tissue (*i.e.*, the tissue appears brighter, or intrinsic signals become negative). For example, data set A is a subsequent averaged image and data set B is an averaged control image. Normally, when a pixel in data set A is subtracted from a pixel in data set B and a negative value results, this value is treated as zero. Hence, difference images cannot account for areas of inhibition. The present invention provides a method for identifying both negative and positive intrinsic signals, by: (a) subtracting data set A (a subsequent averaged image) from data set B (an averaged control image) to create a first difference data set, whereby all negative pixel values are zero; and (b) subtracting data set B from data set A to create a second difference data set whereby all negative pixel values are zero; and adding the first and second difference data sets to create a "sum difference data set." The sum difference data set shows areas of increased activity (*i.e.*, color coded with warmer colors such as yellow, orange, red) and may be visualized as image areas of less activity or inhibition (*i.e.*, color coded with colder colors such as green, blue, purple). Alternatively, one can overlay the first difference data set on the second difference data set. The difference output may be visualized as an image and may be superimposed on the real time analog image to provide an image of the area of interest (*e.g.*, cortical surface) superimposed with a color-coded difference frame to indicate where there are intrinsic signals in response to a challenge, stimulus, paradigm, or the like.

The comparison (*e.g.*, difference) data may be further processed to smooth out the data and remove high frequency noise. For example, a lowpass spatial filter can block high spatial frequencies and/or low spatial frequencies to remove high frequency noises at either end of the dynamic range. This provides a smoothed-out processed difference data set (in digital format). The digitally processed difference data set can be provided as an image and color-coded by assigning a spectrum of colors to differing shades of grey. This image may then be converted back to an analog image (by an ADI board) and displayed for a real time visualization of differences between an averaged control image and subsequent images.

Moreover, the processed difference image can be superimposed over the analog image to display specific tissue sites where a contrast enhancing agent may have a faster uptake, or where an intrinsic signal may be occurring.

Processing speed may be enhanced by adding a real time modular processor or faster CPU chip to the image processor. One example of a real time modular processor which may be employed in the present invention is a 150 RTMP-150 Real Time Modular Processor (Imaging Technology, Woburn, MA). The processor may further include an optical disk for storing digital data, a printer for providing a hard copy of the digital and/or analog data and a display, such as a video monitor, to permit the user to continuously monitor the comparison data output.

A single chassis may house all of the modules necessary to provide optical detection and analysis in a format that can be easily interpreted, such as an image format, according to the present invention. The necessary components, whether or to whatever degree integrated, may be installed on a rack that is easily transportable, along with display monitors and peripheral input and output devices.

A preferred high resolution and high performance system comprising a PentaMAX 576x384FT LCD system (by Princeton Instruments Inc., NJ) digitizes the data at the chip and provides a large dynamic range and reduced noise. This system may be interfaced using a PCI-bus to a dual-400 Mhz Pentium PC running windows NT. Image analysis algorithms may be written in C using Microsoft VisualC++ Version 5.0 compiler. For more rapid online processing, the data may be routed to dedicated imaging hardware residing in the PC computer. For example, IM-PCI hardware (by Imaging Technology Inc., Bedford, MA) could be used. One such configuration would consist of the following IM-PCI boards and modules: IM-PCI, AMVS, and a CMALU.

The imaging methods applied to *in vivo* applications may acquire data at the surface of an area of interest. As described above, longer wavelengths of emr (in the infrared range) can be used to image areas of interest which are deeper in tissue or below overlying tissue. In some areas of the body longer wavelength visible light and near infrared emr can easily pass

through such tissue for imaging. Moreover, if a difference image is created between the image acquired at 500 nm emr and the image acquired at 700 nm emr, the difference image will show an optical slice of tissue. Administration of an imaging agent which absorbs specific wavelengths of emr can act as a tissue filter of emr to provide a filter in the area of interest. In this instance, it is desirable to utilize an imaging agent that remains in the tissue for a prolonged period of time.

In a simple system suitable for assessing cell populations *in vivo* in animal or tissue culture models, the systems of the present invention may include one or more optical fiber(s) operably connected to an emr source that illuminates cells or tissue, and another optical fiber operably connected to an optical detector, such as a photodiode, that detects one or more optical properties of the illuminated cells or tissue. The detector may be used to acquire obtain control data representing the "normal" or "background" optical properties of a sample population, and then to acquire subsequent data representing the optical properties of the sample population during or following administration of a test agent or test condition. A physiological challenge and/or a stimulus that stimulates a disease or pathological state may be administered prior to administration of the treatment agent or condition. The system comprises or is in communication with a data storage and processing system having information storage and processing capability sufficient to compare the geometrical and/or optical properties of individual cells or cell samples to empirically determined standards, or to data acquired at different points in time.

In operation, an area of interest in an *in vitro* or *in vivo* cell sample is illuminated with electromagnetic radiation (emr) and one or a series of data points or data sets representing one or more geometrical and/or optical properties of a spatially resolved area of interest is acquired during an interval of "normal" physiological activity. This data represents a control, or background data profile for that particular cell sample under those particular physiological conditions. A series of data sets is preferably combined, for example by averaging, to obtain a control data profile. The control data profile is stored for comparison with other data sets.

Similarly, control data sets may be collected and stored that represent a background data profile for particular cell types under specified physiological conditions.

Data sets representing the corresponding geometrical and/or optical property of the sample population at the same, spatially resolved areas of interest, are acquired during a subsequent time period. For monitoring applications, data may be collected at regular time intervals to monitor the condition of the cell sample and to detect aberrations from the baseline profile. For screening applications, one or more subsequent data set(s) is collected during a period following physiological activity or inhibition, induced, for example, by introduction of a test compound or by exposure to a test condition. Physiological activity or inhibition may be induced by a "natural" occurrence such as a seizure or stroke in an animal model, or it may be induced by administering a paradigm or an agent to an *in vitro* or *in vivo* cell sample to stimulate changes in geometrical and/or optical properties of the cell sample that are indicative of physiological activity or inhibition. During a monitoring interval or stimulation of an intrinsic physiological response, one or a series of subsequent data sets, representing one or more of the detected geometrical or optical properties of the area of interest, is acquired. A series of subsequent data sets is preferably combined, for example by averaging, to obtain a subsequent data set. The subsequent data set is compared with the control data set to obtain a comparison data set, preferably a difference data set. Comparison data sets are then analyzed for evidence of changes in geometrical and/or optical properties representative of physiological activity or inhibition within the area of interest.

Figure 1 shows a schematic flow diagram illustrating an exemplary system, as well as exemplary output data of the present invention with reference to *in vitro* cell populations. A cell population may comprise cells in suspension at a sparse cell density, or confluent layers of cells, or layers of cells at other predetermined cell densities, or a tissue sample, such as a tissue slice. Maintenance of a wide variety of cell and tissue samples under cell culture conditions is well known in the art.

The sample population is placed at a predetermined location on a platform, such as on a microscope stage. In the example shown in Fig. 1, the sample is an acute rat hippocampal

slice maintained in a submerged perfusion chamber. Alternatively, the sample may be cell samples maintained in cell culture media in flasks, multiple well plates, and the like. Multiple well tissue culture plates may be used for high throughput screening, in combination with an automated stage for positioning cell samples in individual wells for optical detection at predetermined intervals. Programmable, automated positioning devices are well known in the art.

An optical detector (in this case, a CCD camera) is attached to the camera-port of the microscope. During one or more control period(s) and one or more test period(s), data relating to dimensional and/or optical properties of individual cells or of an area of interest in the cell sample are acquired, stored and processed. Acquisition and processing of data may be accomplished as described below and in the Examples.

The grey-scale image on the upper right is the unprocessed image of the tissue-slice as viewed by the CCD camera. This slice was then electrically stimulated at two different intensities: a low-intensity electrical stimulus causing a small increase in neuronal and synaptic activation; and a high-intensity electrical stimulus causing a larger increase in neuronal and synaptic activity.

The pseudo-colored image was generated as described below in Example 1. Briefly, an image acquired during the electrical stimulation was subtracted from an image acquired in the control state. This image was then filtered with a low-pass filter, histogram-stretched, and pseudo-colored. The colors were coded to indicate intensity of activity-evoked optical change (arrow on color-bar).

The dynamic optical changes represented in these images can also be plotted as a graph (lower right image). Here, each data-point represents the average change in light-transmission through the small box indicated on the pseudo-colored image. A series of images, two seconds apart, were acquired and the average value was calculated for each image and plotted as a point on the graph. The tissue was electrically stimulated for two seconds at the points indicated by the straight lines. The small peak indicates the maximum optical change induced by the first small electrical stimulation, the larger peak from the second larger



stimulus. The electrical stimulation was ceased after two seconds and the tissue was allowed to recover. The plots of the recovery are characteristic of the ion-homeostatic mechanisms of the tissue. Their recovery could be quantified, for example, by finding the best exponential fits for the recovery periods.

### **EXAMPLE 1**

Sprague-Dawley rats (male and female; 25 to 35 days old) were prepared as described in Aghajanian, A.K. and Rasmussen, K., *Synapse* 31:331, 1989; and Buckmaster, P.S., Strowbridge, B.W., Schwartzdroin, P.A., *J. Neurophysiol.* 70:1281, 1993. In most hippocampal slice experiments, simultaneous extracellular field electrode recordings were obtained from CA1 and CA3 areas. For stimulation-evoked afterdischarge (13 slices, 8 animals), the concentration of  $Mg^{2+}$  in the bathing medium was reduced to 0.9 mM. A bipolar tungsten stimulating electrode was placed on the Schaffer collaterals to evoke synaptically driven field responses in CA1; stimuli consisted to 100 to 300-  $\mu$ s-duration pulses at an intensity of four times population-spike threshold. Afterdischarges were evoked by a 2-s train of such stimuli delivered at 60 Hz. Spontaneous interictal-like bursts were observed in slices treated with the following modifications or additions to the bathing medium: 10 mM  $K^+$  (6 slices; 4 animals; average, 81 bursts/min), 200 to 300  $\mu$ M 4-AP (4 slices; 2 animals; average, 33 bursts/min), 50 to 100  $\mu$ g M bicuculine (4 slices; 3 animals; average, 14 bursts/min), 0 mM  $Mg^{2+}$  [(1 hour of perfusion) 3 slices; 2 animals; average, 20 bursts/min; (3 hours of perfusion) 2 slices, 2 animals)], 0 mM  $Ca^{2+}$ /6 mM KCl and 2 mM EGTA (four slices, three animals). In all treatments, perfusion with furosemide-containing medium was begun after a consistent level of bursting had been established.

For imaging of intrinsic optical signals, the tissue was illuminated with a beam of white light (tungsten filament light and lens system; Dedotec USA, Lodi, NJ) directed through the microscope condenser. The light was controlled and regulated (power supply: Lambda Electronics, Melville, NY) to minimize fluctuations and filtered (695 nm long-pass) so that the slice was transilluminated with long wavelengths (red). Image frames were acquired with

a charge-coupled device camera (Dage-MTI) at 30 Hz and were digitized at 8 bits with a spatial resolution of 512 by 480 pixels by means of an Imaging Technology Series 151 imaging system; gains and offsets of the camera-control box and the analog-to-digital board were adjusted to optimize the sensitivity of the system. Imaging hardware was controlled by a 486-PC-compatible computer running software written by D. Hochman and developed with commercially available software tools (Microsoft's C/C++ Compiler and Imaging Technology's ITEX library). To increase signal-to-noise ratio, an averaged image was composed from 16 individual image-frames, integrated over 0.5 s and averaged together. An experimental series typically involved the continuous acquisition of a series of averaged images over a several minute time period; at least 10 of these averaged images were acquired as control images before stimulation. Pseudocolored images were calculated by subtracting the first control image from subsequently acquired images and assigned a color lookup table to the pixel values. For these images, usually a linear low-pass filter was used to remove high-frequency noise and a linear-histogram stretch was used to map the pixel values over the dynamic range of the system. All operations on these images were linear so that quantitative information was preserved.

Figures 2A-2C show the effect of the agent furosemide on stimulation evoked afterdischarge activity in a hippocampal tissue slice comparing the field response, measurements at an extracellular electrode, and images highlighting changes in optical properties.

Fig. 2A1 illustrates that two seconds of electrical stimulation at 60 Hz elicited afterdischarge activity. Fig. 2A2 shows a typical afterdischarge episode recorded by the extracellular electrode, with the horizontal arrow indicating the baseline. Fig. 2A3 shows a map of the peak change in optical transmission through the tissue evoked by Schaffer collateral stimulation. The color bar indicates increasing magnitude of activity-evoked optical changes from the bottom to the top of the bar. The region of maximum optical change (red, yellow) corresponds to the apical and basal dendritic regions of CA1 on either side of the stimulating electrode. Fig. 2B1-2BC illustrate responses to electrical stimulation following 20

minutes of perfusion with medium containing 2.5 mM furosemide. Both the electrical afterdischarge activity (shown in Fig. 2B2) and the stimulation-evoked optical changes (shown in Fig. 2BC) were blocked. However, there was a hyperexcitable field response (multiple population spikes) to the test pulse, as illustrated in Fig. 2B1. Figs. 2C1-2C3 illustrate that restoration of the initial response pattern was seen following 45 minutes of perfusion with normal bathing medium.

Figure 3A illustrates an enlarged grey-scale image of an acute rat hippocampal tissue slice, observed using a CCD camera attached to a Zeiss upright microscope. Figures 3B-3E illustrate enlarged, pseudo-colored images acquired as described above. Figure 3B illustrates an enlarged, pseudo-colored image acquired as described above during the peak optical change induced by electrical stimulation, with an enlarged color bar, the arrow on the color bar indicating increasing magnitude of activity-evoked optical changes. The box indicates the field of view shown magnified in Figs. 3C, 3D and 3E. Fig. 3C illustrates the peak optical change during electrical stimulation when no epileptic activity was induced. Fig. 3D illustrates the peak optical change during electrical stimulation that resulted in epileptiform activity. A larger area of increased magnitude of changes in optical properties is observed during epileptiform activity. Fig. 3E illustrates the peak optical change during electrical stimulation following treatment with furosemide, which blocks the epileptiform activity and the intrinsic optical signal.

## **EXAMPLE 2**

This example illustrates optical changes indicative of neuronal activity in a human subject by direct cortical electrical stimulation. Surface electrical recordings (surface EEG, ECOG) were correlated with optical changes. Intrinsic optical changes were evoked in an awake patient during stimulating-electrode "calibration." Four stimulation trials were sequentially applied to the cortical surface, each stimulation evoking an epileptiform afterdischarge episode. A stimulation trial consisted of: (1) monitoring resting cortical activity by observing the output of the recording electrodes for a brief period of time;

(2) applying an electric current via the stimulation-electrodes to the cortical surface at a particular current for several seconds; and (3) monitoring the output of the recording electrodes for a period of time after stimulation has ceased.

The cortex was evenly illuminated by a fiber optic emr passing through a beam splitter, controlled by a D.C. regulated power supply (Lambda, Inc.) and passed through a 695 nm longpass filter. Images were acquired with a CCD camera (COHU 6500) fitted to the operating microscope with a specially modified cineadapter. The cortex was stabilized with a glass footplate. Images were acquired at 30 Hz and digitized at 8 bits (512 x 480 pixels, using an Imaging Technology Inc. Series 151 system, Woburn, MA). Geometric transformations were applied to images to compensate for small amounts of patient motion (Wohlberg, *Digital Imaging Warping*, IEEE Computer Society: Los Alamitos, CA, 1988). Subtraction of images collected during the stimulated state (*e.g.*, during cortical surface stimulation, tongue movement or naming) from those collected during a control state with subsequent division by a control image resulted in percentage difference maps. Raw data (*i.e.*, no digital enhancement) were used for determining the average optical change in specified regions (average sized boxes was 30 x 30 pixels or 150-250  $\mu\text{m}^2$ ). For pseudocolor images, a linear low pass filter removed high frequency noise and linear histogram transformations were applied. Noise was defined as the standard deviation of fluctuations in sequentially acquired control images as 0.003-0.009.

A series of images (each image consisting of an average of 128 frames acquired at 30 Hz) were acquired during each of the four stimulation trials. A current of 6 mA was used for the first three stimulation trials, and 8 mA for the fourth. After a sequence of 3-6 averaged control images were acquired, a bipolar cortical stimulation current was applied (either 6 mA or 8 mA) until epileptiform after discharge activity was evoked (as recorded by the surface electrode). Images were continuously acquired throughout each of the four stimulation trials.

The percentage change in absorption of light for each pixel was calculated for each image acquired during the four stimulation trials. The average percentage changes over the four areas (indicated by the four square regions marked in Figure 4A) were plotted graphically

in Figures 4B, 4C, and 4D for comparison and analysis of the dynamic changes occurring in these four spatial areas.

The optical changes between the stimulating electrodes (Site #1, Fig. 4A) and near the recording electrode (Site #3) showed a graded response to the intensity and duration of each afterdischarge episode (Figure 4B). The spatial extent of the epileptiform activity was demonstrated by comparing a baseline image collected before stimulation to those obtained immediately after stimulation. The intensity and spread of the optical changes were much less following Stimulation #2 (shortest, least intense afterdischarge episode) than after Stimulation #4 (longest, most intense afterdischarge episode).

When the optical changes were below baseline, the surface EEG recordings did not identify epileptiform activity ( $n = 3$  patients). At Site #3, the optical changes after stimulation were below baseline. However, during the fourth stimulation, the epileptiform activity spread into the area of Site #3 and the optical signal did not go below baseline until later. This negative optical signal likely represents inhibited neuronal populations (an epileptic inhibitory surround), decreased oxygen delivery, or blood volume shunted to activated regions.

Figure 4B shows plots of the percent optical change per second in the spatial regions of Boxes 1 and 3 (as labeled in Figure 4A). For both regions, the peak change is during the fourth stimulation trial (at 8 mA), in which the greatest amount of stimulating current had induced the most prolonged epileptiform afterdischarge activity. The changes within Box 3 were greater and more prolonged than those of Box 1. Box 3 was overlying the area of the epileptic focus.

Figure 4C shows plots of the percent optical change per second in the spatial regions of Boxes 1 and 4 (as labeled in Figure 4A). Box 1 overlays an area of cortical tissue between the two stimulating electrodes, and Box 4 overlays a blood vessel. The optical changes within box 4 are much larger and in the opposite direction of Box 1. Also, these changes are graded with the magnitude of stimulating current and afterdischarge activity. The changes in Box 4 are most likely due to changes of the blood-flow rate within a blood vessel.

Figure 4D shows plots of the percent optical change absorption per second in the spatial regions of boxes 1 and 2 (as labeled in Figure 4A). Note that although these two areas are nearby each other, their optical changes are in the opposite direction during the first three stimulation trials using 6 mA current. The negative going changes within the region of Box 2 indicate that the methods and apparatus of the present invention may be used to monitor inhibition of physiological changes, as well as excitation.

Figure 5 shows percentage difference images representative of various times during two of the stimulation trials described above. The top three images (5A2, 5B2, and 5C2) are from Stimulation Trial 2, where 6 mA cortical stimulation evoked a brief period of afterdischarge. These are compared to the bottom three images (5A4, 5B4, and 5C4), which are from Stimulation Trial 4, showing the optical changes evoked by cortical stimulation at 8 mA. Figures 5A2 and 5A4 compare control images during rest. Figures 5B2 and 5B4 compare the peak optical changes occurring during the epileptiform afterdischarge activity. Figures 5C2 and 5C4 compare the degree of recovery 20 seconds after the peak optical changes were observed. The magnitude of optical change is indicated by the grey-scale changes. Each image maps an area of cortex approximately 4 cm by 4 cm.

Figures 6A-6H show eight percentage difference images from Stimulation Trial 2, as described above. Each image is integrated over a two second interval. The focal area of greatest optical change is in the center of images 3C, 3D, and 3E, indicating the region of greatest cortical activity. This region is the epileptic focus. The magnitude of optical change is indicated by the grey-scale bar on the right side of the Figure. The arrow beside this grey-scale indicates the direction of increasing amplitude. Each image maps an area of cortex approximately 4 cm by 4cm.

Figures 7A-7H illustrate a real-time sequence of dynamic changes of stimulation-evoked optical changes in human cortex. Figure 4, panels 4A through 4H, show eight consecutive percentage difference images. Each image is an average of 8 frames (  $<1/4$  second per image). The magnitude of optical change is indicated by the grey-scale changes. Each image maps to an area of cortex that is approximately 4 cm by 4 cm. This figure

demonstrates that the methods and apparatus of the present invention can be used to acquire, in real time, data reflecting dynamic changes of optical properties that reflect physiological changes.

### **EXAMPLE 3**

Stimulation mapping of the cortical surface was performed on awake human patients under local anesthesia to identify sensory/motor cortex and Broca's areas. The illumination source and optical detection device and processing techniques used were the same as those described in Example 2. During three "tongue wiggling" trials, images were averaged (32 frames, 1 sec) and stored every 2 seconds. A tongue wiggling trial consisted of acquiring 5-6 images during rest, then acquiring images during the 40 seconds that the patient was required to wiggle his tongue against the roof of his mouth, and then to continue acquiring images during a recovery period. The same patient was then required to engage in a "language naming" trial. A language naming trial consisted of acquiring 5-8 images during rest (control images – the patient silently viewing a series of blank slides), then acquiring images during the period of time that the patient engaged in the naming paradigm (naming a series of objects presented with a slide projector every 2 seconds, selected to evoke a large response in Broca's area), and finally a series of images during the recovery period following the time when the patient ceased his naming task (again viewing blank slides while remaining silent). The results are shown in Figs. 8 and 9.

Figures 8A1-8BC illustrate functional mapping of human language (Broca's area) and tongue and palate sensory areas in an awake human patient as described in Example 3. Images 8A1 and 8B1 are grey-scale images of an area of human cortex, with left being anterior, right-posterior, top-superior, and the Sylvian fissure on the bottom. The two asterisks on 8A1, 8B1, 8A2, and 8B2 serve as reference points for these images. The scale bars in the lower right corner of 8A1 and 8B1 are equal to 1 cm. In 8A1, the numbered boxes represent sites where cortical stimulation with electrical stimulating electrodes evoked palate tingling (1), tongue tingling (2), speech arrest-Broca's areas (3,4) and no response (11, 12, 17, 5, 6-7

premotor). Image 8A2 is a percentage difference control image of the cortex during rest in one of the tongue wiggling trials. The grey-scale bar on the right of 8A2 shows the relative magnitude of the grey values associated with images 8A2, 8A3, 8B2 and 8B3. Image 8A3 is a percentage difference map of the peak optical changes occurring during one of the tongue wiggling trials. Areas identified as tongue and palate sensory areas by cortical stimulation showed a large positive change. Suppression of baseline noise in surrounding areas indicated that, during the tongue wiggling trials, language-motor areas showed a negative-going optical signal. Image 8B2 is percentage difference control image of the cortex during one of the language naming trials. Image 8B3 is a percentage difference image of the peak optical change in the cortex during the language naming task. Large positive-going signals are present in Broca's area. Negative-going signals are present in tongue and palate sensory areas.

Figure 9 shows time course and magnitude plots of dynamic optical changes in human cortex evoked in tongue and palate sensory areas and in Broca's area (language). This figure shows the plots of the percentage change in the optical absorption of the tissue within the boxed regions shown in Figure 8, images 8A1 and 8B1, during each of the three tongue wiggling trials and one of the language naming trials (see description of Figure 8). Figure 9A shows the plots during the three tongue wiggling trials averaged spatially within the Boxes 1, 2, 3, and 4 as identified in Figure 8A1. Figure 9B shows the plots during one of the language naming trials averaged spatially within the Boxes 1-7 and 17.

These results agree with those data reported by Lee, et al. (*Ann. Neurol.* 20:32, 1986), who reported large electrical potentials in the sensory cortex during finger movement. The magnitude of the optical changes in the sensory cortex during tongue movement (10-30%) parallels sensory/motor cortex studies where cerebral blood flow increases 10-30% during motor tasks (Colebatch et al., *J. Neurophysiol.* 65:1392, 1991). Further, utilizing Magnetic Resonance Imaging (MRI) of blood volume changes in human visual cortex during visual stimulation, investigators have demonstrated increases of up to 30% in cerebral blood volume (Belliveau et al., *Science* 254:716, 1991).



Optical images were obtained from this same cortical region (*i.e.*, area of interest) while the patient viewed blank slides and while naming objects on slides presented every two seconds. Percentage difference maps obtained during naming showed activation of the premotor area. The sites of speech arrest and palate tingling were identified by surface stimulation and demonstrate optical signals going in the opposite direction. The area of activation was clearly different from that evoked by tongue movement without speech production. The optical images of premotor cortex activation during naming were in similar locations to the cortical areas identified in PET single word processing studies (Peterson, et al., *Nature* 331:585, 1991; and Frith et al., *J. Neuropsychologia* 29:1137, 1991). The optical changes were greatest in the area of the cortex traditionally defined as Broca's area and not in areas where electrical stimulation caused speech arrest.

#### **EXAMPLE 4**

Areas of interest can be imaged through intact tissues, such as bone, dura, muscle, connective tissue and the like. Figs. 10A-10D illustrate identification of a brain tumor through the intact cranium using optical imaging techniques of the present invention.

Figure 10A is a grey-scale image of the cranial surface of a rat. The sagittal suture runs down the center of the image. Box 1 lays over the suspected region of brain tumor, and Box 2 lays over normal tissue. Figure 10B is a difference image one second after indocyanine green dye had been intravenously injected into the animal. The region containing tumor tissue became immediately visible through the intact cranium. Figure 10C shows that five seconds after dye injection the dye can be seen to profuse through both normal and tumor tissue. Figure 10D shows that one minute after dye injection, the normal tissue had cleared the dye, but dye was still retained in the tumor region. The concentration of dye in the center of this difference image was dye circulating in the sagittal sinus. Dynamic changes in optical properties of cell populations and tissue may likewise be imaged *in vivo* through other intact tissues, such as bone, dura, muscle, connective tissue, and the like.

I Claim:

1. A method for assessing the physiological condition of a biological material, comprising:

maintaining at least one sample population of the biological material in one of the following systems: a cell culture system; a tissue culture system; an organ culture system; and an intact organism;

acquiring test data relating to one or more geometrical or optical properties of the sample population; and

comparing the test data acquired to comparison data relating to one or more geometrical or optical properties of a comparison cell population, the comparison data representing one or more geometrical or optical properties of the comparison cell population in a predetermined physiological state, whereby changes in the one or more geometrical or optical properties reflected in the test data compared to the comparison data represent changes in the physiological state of the sample population.

2. A method of claim 1, additionally comprising acquiring multiple test data sets relating to one or more geometrical or optical properties at multiple, predetermined spatial locations in the sample population.

3. A method of claim 1, additionally comprising exposing the sample population to a physiological challenge prior to acquiring the test data.

4. A method of claim 3, additionally comprising acquiring control data relating to the one or more geometrical properties of the sample population prior to exposing the sample population to a physiological challenge.

5. A method of claim 4, additionally comprising comparing the test data to the control data to assess changes in the one or more geometrical or optical properties of the sample population representing changes in the physiological state of the sample population.

6. A method of claim 1, wherein the comparison data is derived from empirically determined controls.

7. A method of claim 3, wherein the physiological challenge is selected from the group consisting of: exposure to a test agent, a test condition, a drug, a hormone, a biological agent, a toxin, an infectious agent, radiation, chemotherapy, deprivation of a metabolite or nutrient, electrical stimulation, inflammatory agent, oncogen.

8. A method of claim 1, additionally comprising maintaining multiple sample populations in an *in vitro* culture system.

9. A method of claim 1, wherein the test data acquired relates to one or more optical properties selected from the group consisting of: reflection, refraction, diffraction, absorption, scattering, birefringence, refractive index and Kerr effect.

10. A method according to claim 1, wherein the biological material is a viable sample population maintained in a cell culture system.

11. A method according to claim 1, wherein the biological material is a viable, intact organism.

12. A system for assessing the physiological condition of a biological material, comprising:

a platform for supporting an optically transparent container of biological material;

an illumination source for illuminating the biological material;

an optical detector for acquiring test data relating to a geometrical or optical property of the biological material; and

a data storage and analysis device for storing data relating to a geometrical or optical property of the biological material, and comparing test data with a control data profile to generate a comparison data set relating to geometrical or optical property of the biological material;

13. A system of claim 12, wherein the platform is an automated stage operated by a control device capable of locating and moving the stage to predetermined x-y coordinates.

14. A system of claim 12, additionally comprising a data output device capable of displaying the comparison data in a visual format.

15. A system of claim 14, wherein the data output device is capable of displaying comparison data in a graphical or image format.

16. A system of claim 12, wherein the optical detector is a charge coupled device (CCD).

17. A method for identifying optical contrast enhancing agents useful for enhancing the sensitivity of optical detection of a biological material comprising:

maintaining at least one sample population of the biological material in one of the following systems: a cell culture system; a tissue culture system; an organ culture system; and an intact organism;

exposing the at least one sample population to a candidate optical contrast enhancing agent;

acquiring test data relating to one or more optical properties of the sample population subsequent to exposure to the candidate optical contrast enhancing agent; and

comparing the test data acquired to comparison data relating to the one or more optical properties of the sample population.

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**METHODS AND SYSTEMS FOR ASSESSING BIOLOGICAL MATERIALS USING  
OPTICAL DETECTION TECHNIQUES**

5

**Abstract of the Disclosure**

Optical detection techniques for the assessment of the physiological state, health and/or viability of biological materials are provided. Biological materials which may be examined using such techniques include cells, tissues, organs and subcellular components. The inventive techniques may be employed in high throughput screening of potential diagnostic and/or therapeutic agents.

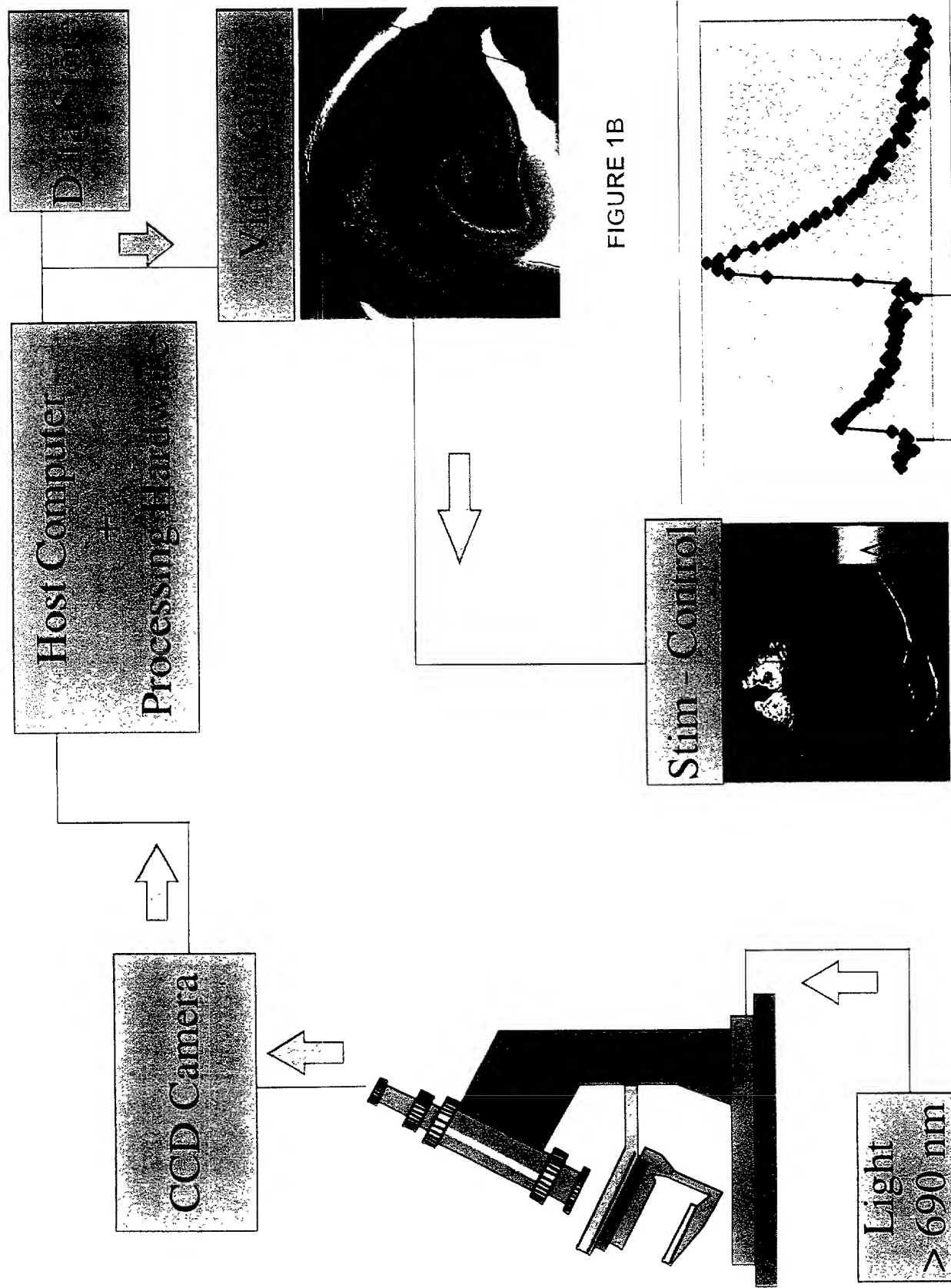
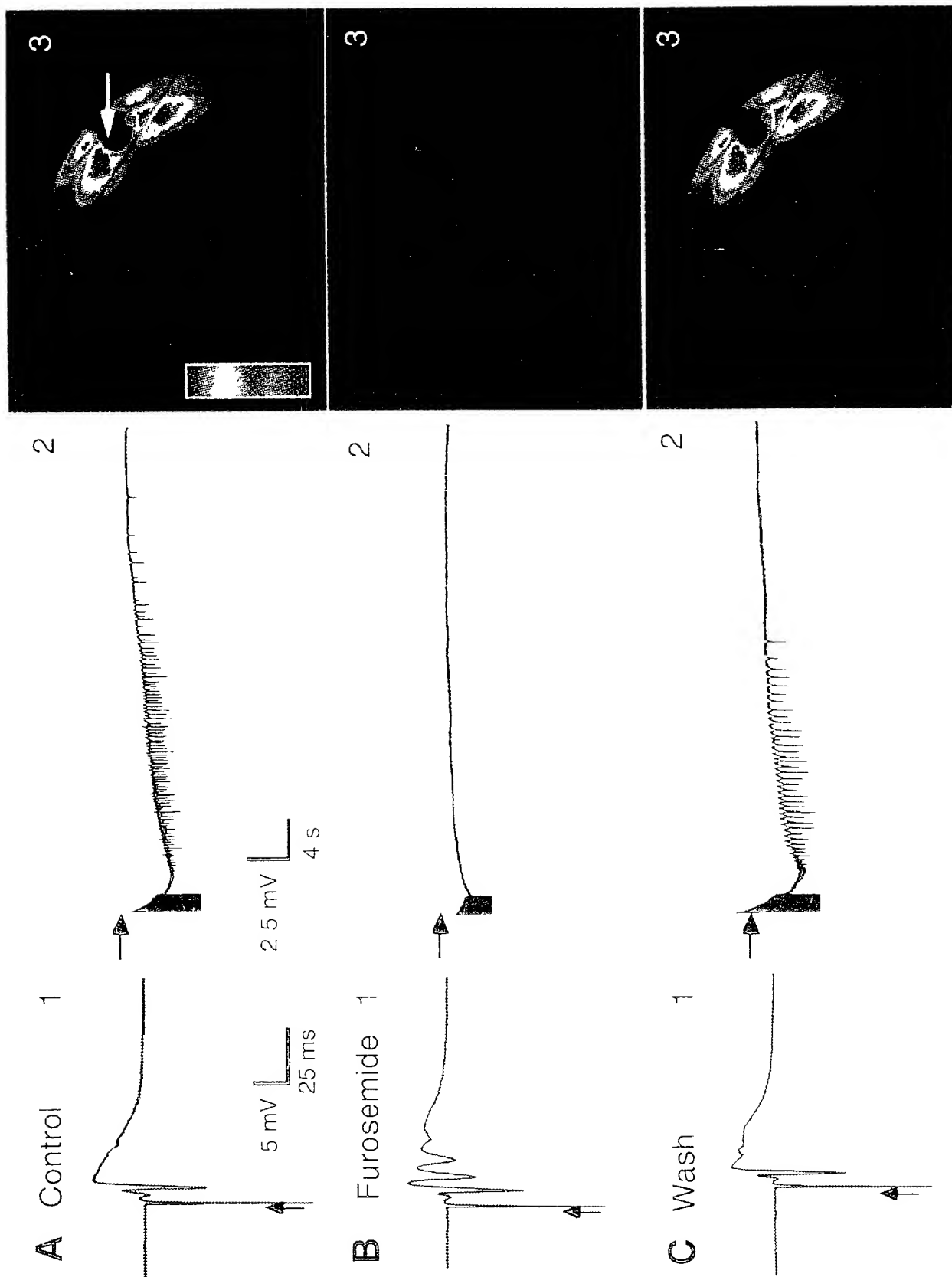


FIGURE 1A

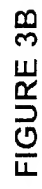
FIGURE 1C

FIGURE 1B

FIGURE 1D





[illegible]

**FIGURE 3D**

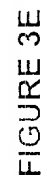


FIGURE 4A

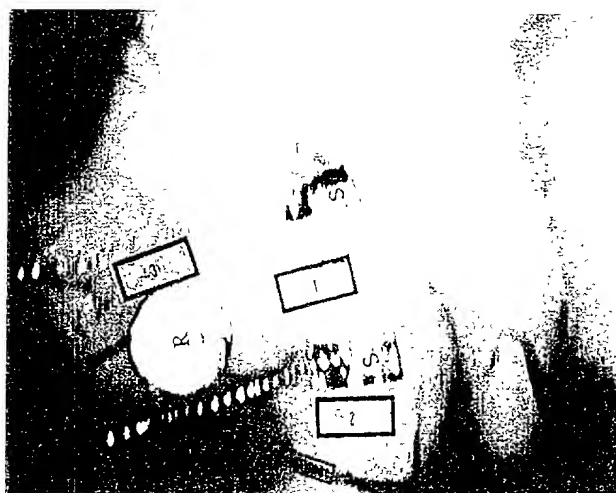


FIGURE 4B

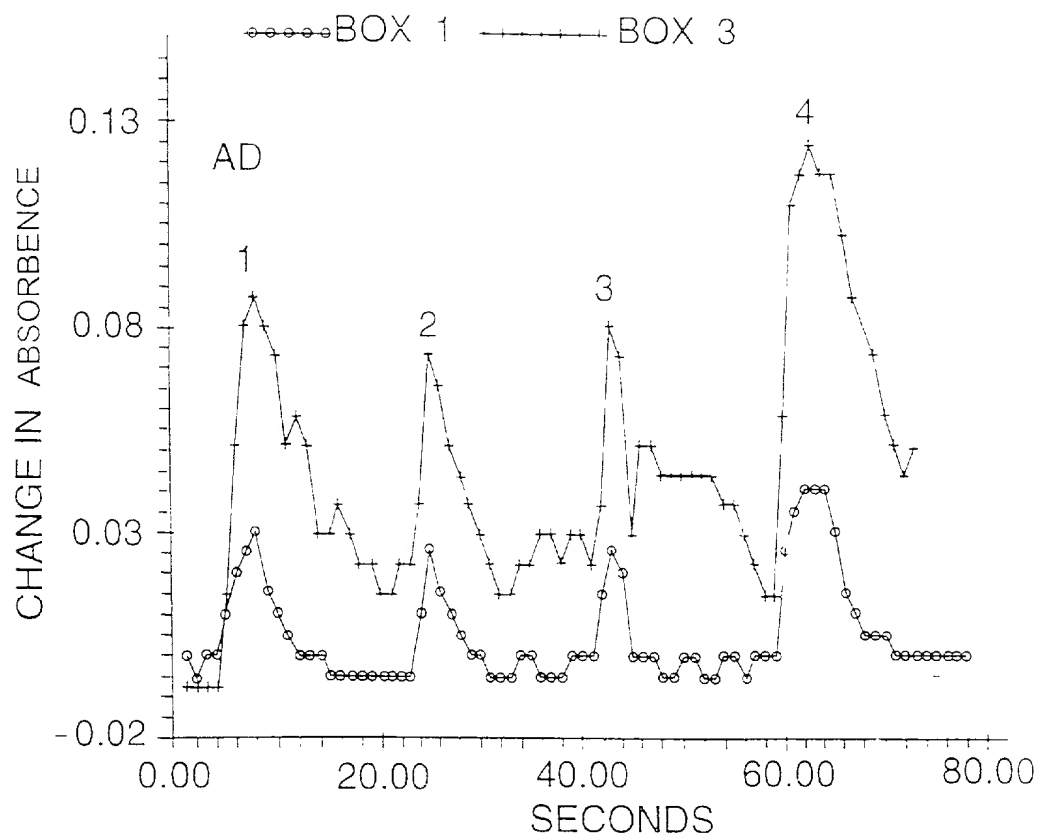


FIGURE 4C

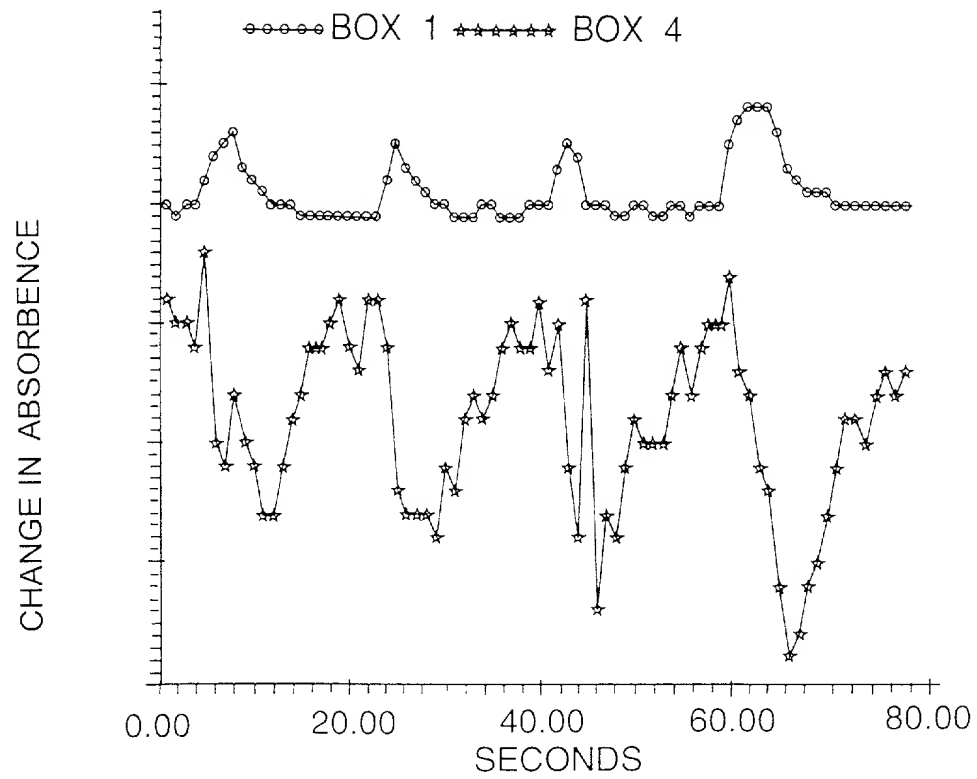


FIGURE 4D

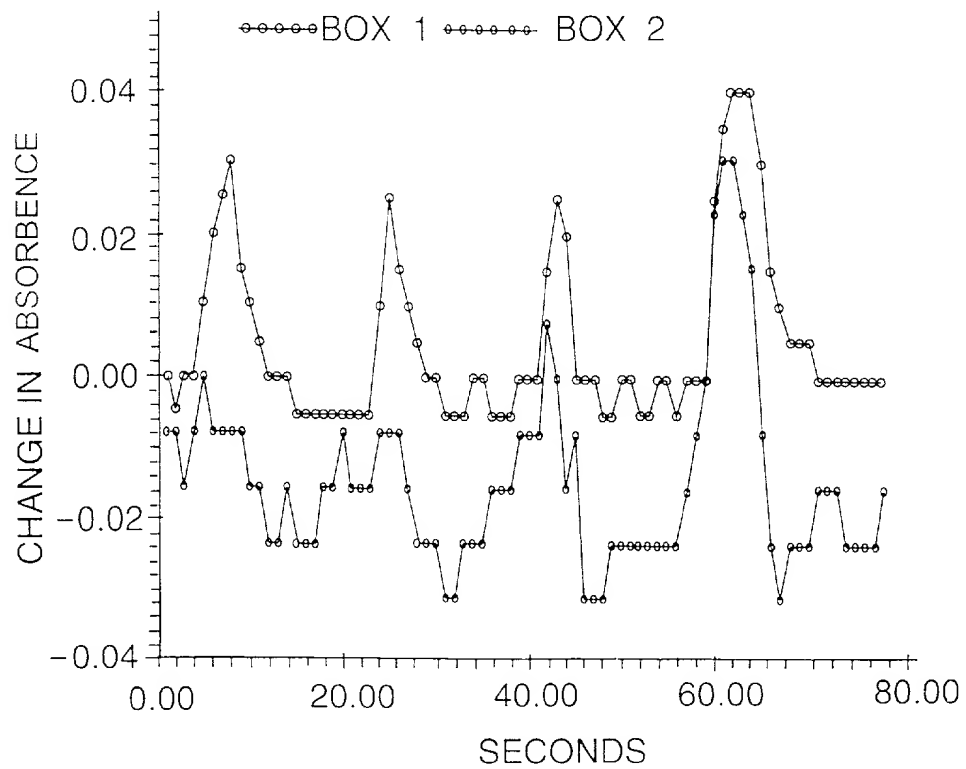


FIGURE 5A2



FIGURE 5B2



FIGURE 5C2



FIGURE 5A4



FIGURE 5B4



FIGURE 5C4





FIGURE 7A



FIGURE 7B



FIGURE 7C



FIGURE 7D



FIGURE 7E



FIGURE 7F



FIGURE 7G



FIGURE 7H



FIGURE 8A1

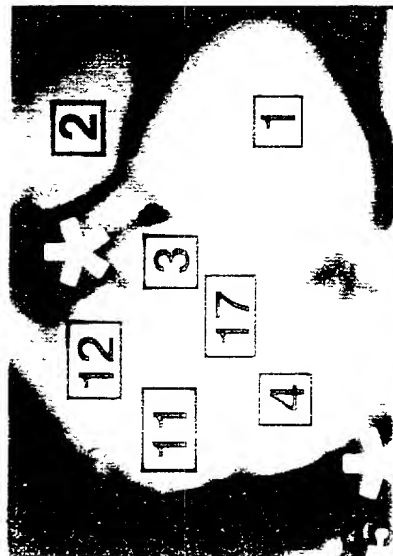


FIGURE 8A2



FIGURE 8A3

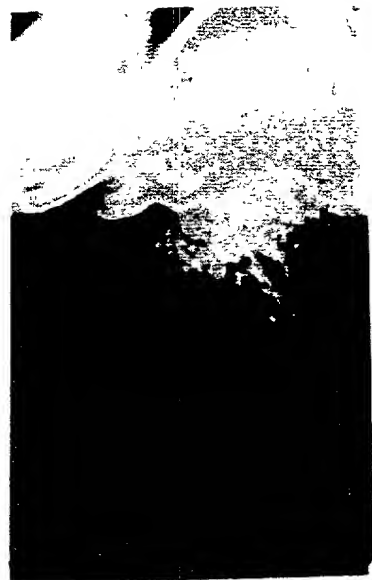


FIGURE 8B1

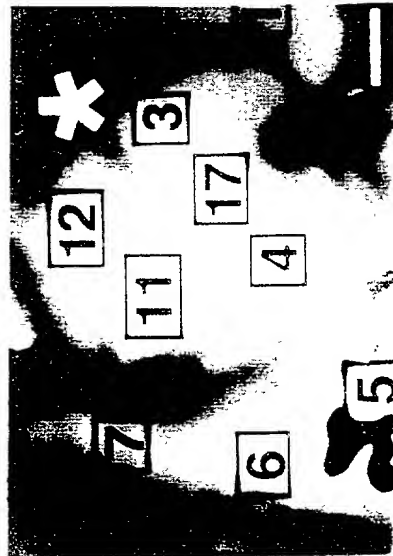


FIGURE 8B2

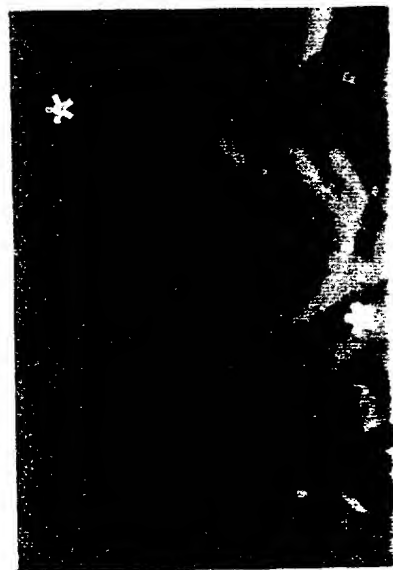


FIGURE 8B3



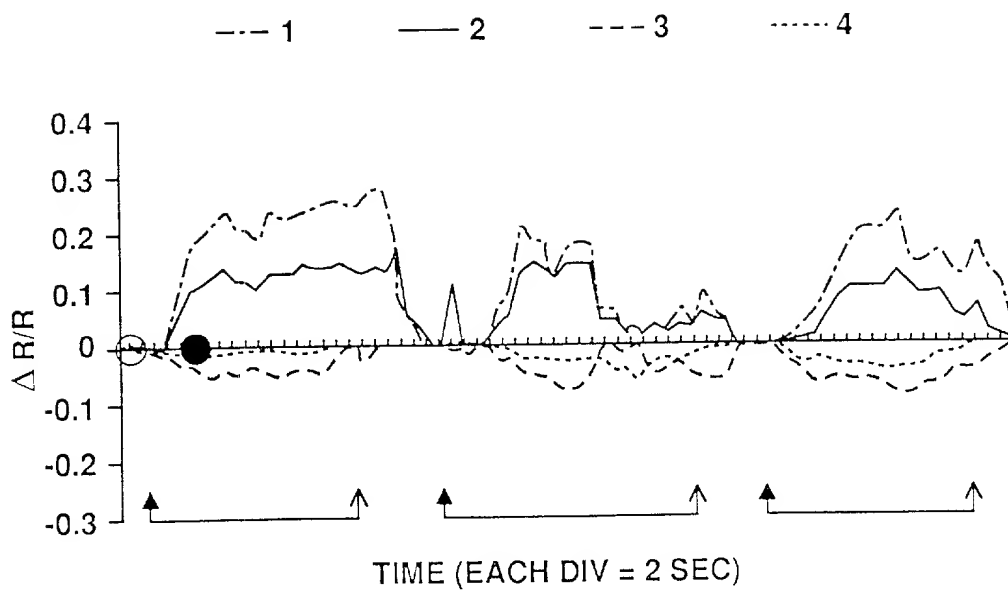


FIGURE 9A

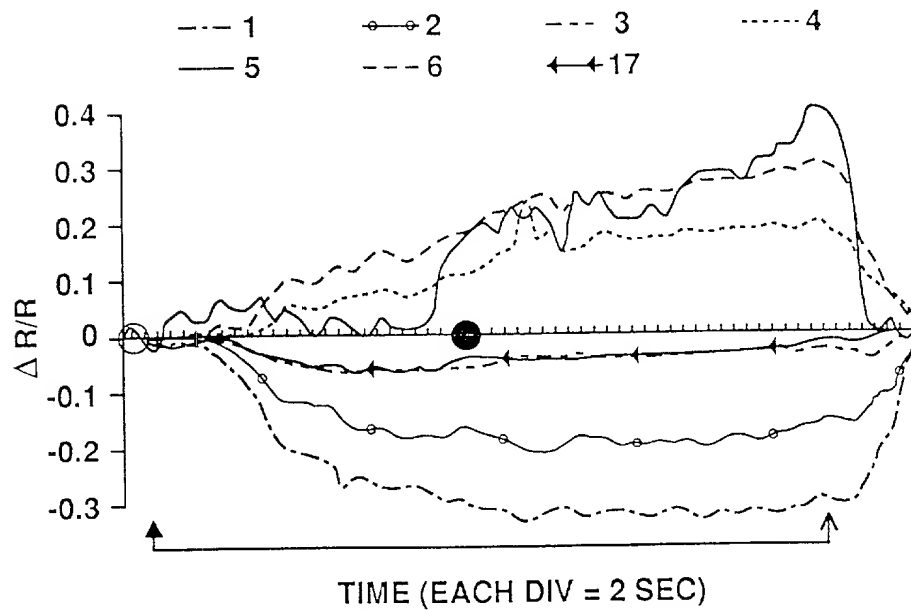


FIGURE 9B



FIGURE 10A



FIGURE 10B

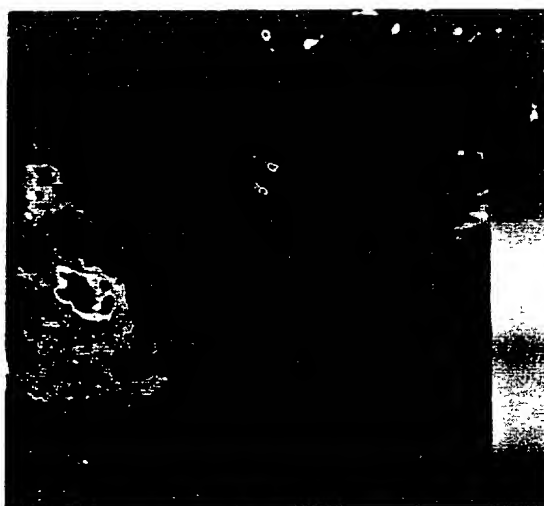


FIGURE 10C

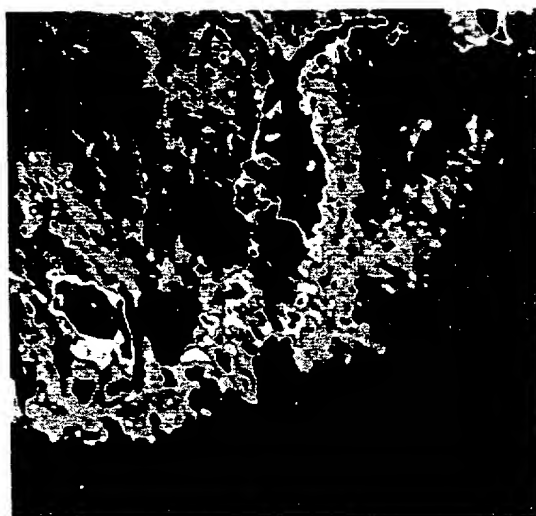


FIGURE 10D

**COMBINED DECLARATION AND POWER OF ATTORNEY**  
**FOR PATENT APPLICATION**

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled **METHODS AND SYSTEMS FOR ASSESSING BIOLOGICAL MATERIALS USING OPTICAL DETECTION TECHNIQUES**, the specification of which

☒ is attached hereto.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed: **NONE**

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application: **U.S. Patent Application No. 60/088,494, filed June 8, 1998.**

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

<u>Name</u>	<u>Reg. No.</u>
Ann W. Speckman	31,881
Janet Sleath	37,007
Jim Klaniecki	38,207

Direct all telephone calls to Ann W. Speckman at telephone number:  
(206) 269-0565.

Address all correspondence to Ann W. Speckman at:

LAW OFFICES OF ANN W. SPECKMAN  
2601 Elliott Avenue, Suite 4185  
Seattle, Washington 98121

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole inventor: Daryl W. Hochman

Inventor's signature: \_\_\_\_\_ Date: \_\_\_\_\_

Residence : Seattle, Washington USA

Citizenship : USA

Residence address : 337 NE 56<sup>th</sup>  
Seattle, Washington 98105